Amorphigenin inhibits Osteoclast differentiation by suppressing c-Fos and nuclear factor of activated T cells

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Abstract: Among the several rotenoids, amorphigenin is isolated from the leaves of *Amopha Fruticosa* and it is known that has anti-proliferative effects and anti-cnacer effects in many cell types. The main aim of this study was to investigate the effects of amorphigenin on osteoclast differentiation in vitro and on LPS treated inflammatory bone loss model in vivo. We show here that amorphigenin inhibited RANKL-induced osteoclast differentiation from bone marrow macrophages in a dose dependent manner without cellular toxicity. Anti-osteoclastogenic properties of amorphigenin were based on a down-regulation of c-fos and NFATc1. Amorphigenin markedly inhibited RANKL-induced p38 and NF-κB pathways, but other pathways were not affected. Micro-CT analysis of the femurs showed that amorphigenin protected the LPS-induced bone loss. We concluded that amorphigenin can prevent inflammation-induced bone loss. Thus we expect that amorphigenin could be a treatment option for bone erosion caused by inflammation.

Introduction

Osteoclasts are bone resorbing multinuclear cells originating from the hematopoietic stem cells. They attach to the exterior of the bone and erode the surface with numerous enzymes and acids (Teitelbaum 2000; Boyle *et al.,* 2003). The main role of osteoclasts is bone remodeling, they constantly dissolve and get rid of old or damaged bones making way for the osteoblasts make new ones. Decrease in bone mass resulting from imbalance between bone resorption by osteoclasts and bone formation by osteoblasts can be caused by various hormones and cytokines, which often leads to bone diseases such as autoimmune arthritis, periodontitis, postmenopausal osteoporosis, paget's disease, and bone tumors (Rodan & Martin, 2000; Takayanagi *et al.,* 2000).

The first line treatment that are currently used in treating osteoporosis primarily include bisphosphonates (e.g. alendronate, risedronate), along with estrogen replacement therapy, selective estrogen receptor modulators (SERM), and calcitonin for postmenopausal osteoporosis. However, such therapies are associated with adverse effects, including breast cancer, endometritis, thromboembolism, hypercalcemia, GI problems, and hypertension (Lloyd 1998; Rodan & Martin, 2000; O'Regan & Gradishar, 2001; Body 2002; Watts 2003).

Most of the drugs we use to treat osteoporosis have many side effects. Thus, many plant-derived natural products have

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been used in traditional medicine for the treatment of various diseases. Recently, several previous reports have shown that several compounds derived from natural products suppress osteoclast differentiation and function (Li *et al.,* 2007; Hu *et al.,* 2008). Also, we have previously reported that curcumin, a compound derived from *Curcuma aromatica*, *Stewartia koreana* extract, tanshinone IIA derived from Danshen (also called Tanshen, the deried roots of *Salvia miltiorrhiza*) exert an inhibitory effect on osteoclast differentiation and bone resorption in vitro and in vivo (Bharti *et al.,* 2004; Kwak *et al.,* 2006; Park *et al.,* 2007). More recently, we reported that rotenone, which is derived from Derris elliptica, significantly inhibits osteoclast differentiation, bone resorption, and bone erosion (Kwak *et al.,* 2010). Thus, we screened rotenone derivate and examined the effect of amorphigenin in osteoclast differentiation and bone resorption in vitro and in vivo. Amorphigenin (also known as 8'-hydroxyrotenone), an aglycone of the rotenoid glycoside amorphin from plants of the genus *Amorpha*, was initially identified as a flavonoids isolated from *Amorpha fructiosa* (Kloutek *et al.,* 1985). Up to now little is known about the biological function of amorphigenin.

In this study, we found that amorphigenin suppressed osteoclast differentiation of osteoclast precursors, bone marrow-derived macrophages (BMMs). The effect of amorphigenin may be involved to the inhibition of c-Fos and NFATc1 expression in BMMs treated RANKL. Amorphigenin considerably inhibits bone erosion in a mouse model of bone loss.

Materials and Methods

Reagents and antibodies

Amorphogen, TRAP assay kit, LPS, and anti-actin anti-body were purchased from Sigma Aldrich (St. Louis, MO, USA). Human soluble RANKL and M-CSF were from PeproTech EC (London, UK). For cytotoxicity test, XTT assay kit was purchased from Roche Diagnostics (Indianapolis, IN, USA). Antibodies against c-Fos and NFATc1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Phospho (p)-p38, p-JNK, p-ERK, p-Akt, Akt, I-κB was purchased from Cell signaling Technology (Beverly, MA). ICR mice were from Dae Han Biolink Co. Ltd. (Chungbuk, Korea).

Osteoclast differentiation

Bone marrow cells (BMCs) were isolated from long bones of 5-week-old ICR mouse by flushing with α-minimum essential medium (α-MEM; Welgene, Daegu, Korea) containing antibiotics (Sigma Aldrich), and further red blood cells (RBC) were removed with RBC lysis buffer (Sigma Aldrich). The cells were placed on 90-mm culture dishes (Corning, NY, USA) and incubated for 1 day in α-MEM containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and antibiotics (Gibco BRL) in the presence of M-CSF (10 ng/ml). Nonadherent cells were seeded on 90-mm petri dishes and then cultured for 3 days in the presence of M-CSF (30 ng/ml). Adherent cells were used as bone marrow-derived macrophages (BMMs), namely osteoclast precursors. To generate osteoclasts from osteoclast precursors, BMMs were further cultured for 4 days in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml) with or without amorphigenin in various concentrations.

RT-PCR

Total RNA was isolated from treated cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using oligo dT, dNTP, dithiothreitol, RNase inhibitor, 1x PCR buffer, and Superscript II reverse transcriptase (Invitrogen). 1 μl of cDNA was amplified by PCR using Taq polymerase (Invitrogen). The following primers were used: c-Fos sense, 5'-CTGGTGCAGCCCACTCTGGTC-3'; c-Fos antisense, 5'-CTTTCAGCAGATTGGCAATCTC-3'; NFATc1 sense, 5'-CAACGCCCTGACCACCGATAG-3'; N FATc1 antisense, 5'-GGCTGCCTTCCGTCTCATAGT-3'; TRAP sense, 5'-ACTTCCCAGCCCTTACTAC-3'; TRAP antisense, 5'-TCAGCACATAGCCCACACCG-3'; GAPD H sense, 5'-ACCACAGTCCATGCCATCAC-3'; OSCAR sense, 5'-CTGCTGGTAACGGATCAGCTCCCCAGA-3'; OSCAR antisense, 5'-CCAAGGAGCCAGAACCTTCGAAACT-3'; GAPDH antisense, 5'-TCCACCACCCTGTTGCTGTA-3'. PCR products were electrophoresed on 1% agarose gels and visualized under UV after ethidium bromide staining.

Western blotting

Cells were lysed in lysis buffer containing 50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. Proteins were boiled in SDS sample buffer,

resolved by 10% SDS-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was probed with antibodies, as indicated, washed extensively, and then incubated with secondary antibody conjugated to horseradish peroxidase. Bound antibody was detected by using enhanced chemiluminescent reagents (ECL plus, GE Healthcare).

Cytotoxicity assay

BMMs (1×10^4 cells/well) were seeded in triplicate in 96-well plates in α-MEM and then incubated for 3 days with M-CSF (30 ng/ml) in the presence or absence of amorphigenin in various concentrations. After 3 days incubation, 50 μl of XTT reagents were added to each well and incubated for 4 h. Absorbance was measured at 450 nm using an ELISA plate reader (Thermomax; Molecular Devices, Sunnyvale, CA).

LPS-induced bone erosion

6-week-old ICR mice were intraperitoneally administered amorphigenin (10 μg/g of body weight) or PBS 1 day before the injection of LPS. Amorphigenin was intraperitoneally administered every other day for 8 days. Mice were injected intraperitoneally with LPS (5 μg/g of body weight) on days 0 and 4. The femurs were obtained on the day 8 after the first injection of LPS and were fixed in 4% paraformaldehyde for 1 day. Radiographic images were taken with micro-CT scan (NFR-Polaris-S160: Nano Focus Ray, Iksan, Korea). Bone volume/tissue volume was calculated by using the VG Studio program.

Statistical analysis

All quantitative results are expressed as mean±S.D. from at least 3 independent experiments. Differences among groups were assessed using the one-factor analysis of variance (ANOVA) followed by Duncan's multiple range test. Values of $P < 0.05$ were considered as statistically significant and expressed as asterisk.

Results

Amorphigenin inhibits RANKL-mediated osteoclast differentiation

Because rotenone significantly inhibits osteoclast differentiation and bone erosion in vitro and in vivo as reported

previously (Kwak *et al.,* 2010), we examined whether amorphigenin, a rotenone derivate, can inhibit osteoclast differentiaion. Osteoclasts were differentiated from mouse BMMs in the presence of M-CSF (30 ng/ml) plus RANKL (50 ng/ml). The BMMs of the control group differentiated into mature TRAP-positive multinucleated osteoclasts while amorphigenin reduced the formation and numbers of TRAP-positive multinucleated cells in a dose-dependent manner
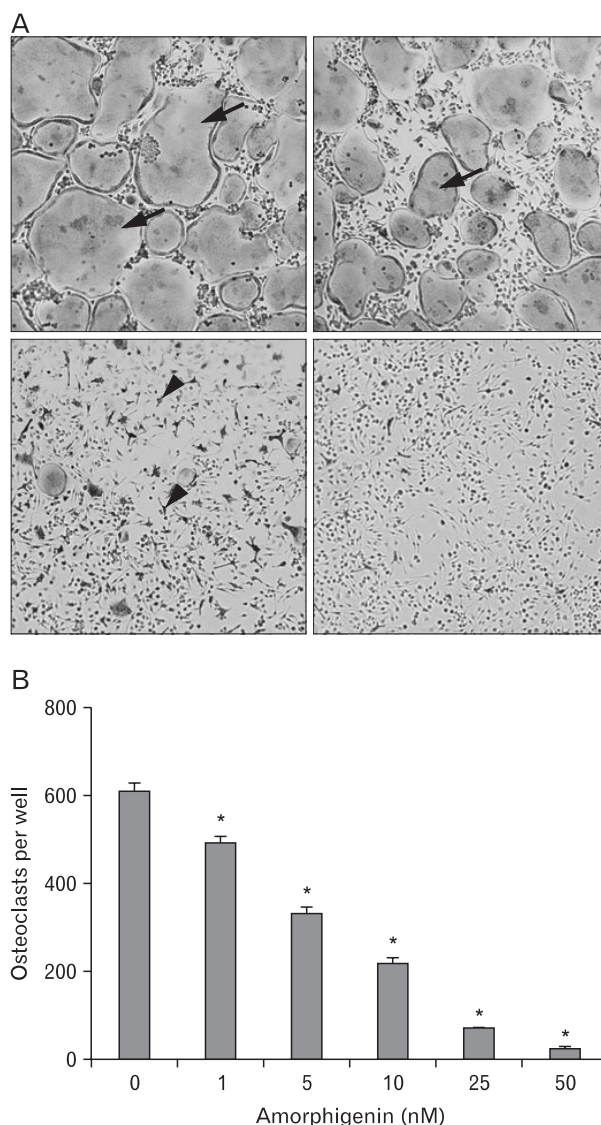

Fig. 1. Amorphigenin inhibits osteoclast differentiation in BMMs. (A) BMMs were cultured with M-CSF (30 ng/ml) and RANKL (50 ng/ml) in the presence of amorphigenin for 4 days. Then the cells were fixed in 3.7% formalin, permeabilized in 0.1% Triton X-100, and stained for TRAP. Arrows indicate the multinuclear osteoclasts. Arrowheads indicate the mononuclear osteoclasts (B) TRAP-positive cells were counted as osteoclasts. Askerisks indicate statistical difference from the control.
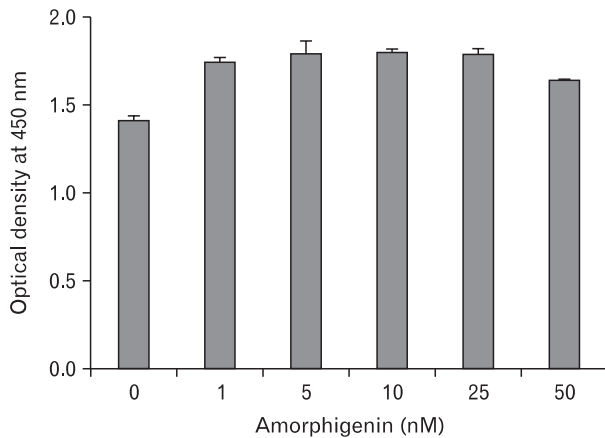
Fig. 2. Effect of amorphigenin on cytotoxicity. BMMs were seded onto 96-well plate. Then the cells were cultured for 3 days with the indicated concentration of amorphigenin in the presence of M-CSF (30 ng/ml). After 3 days, XTT reagents were added to each well. The optical density was read at 450 nm in an ELISA plate reader after 4 hours of incubation. Cell viability was determined relative to the control.

(Fig. 1).

The cytotoxic effect of amorphigenin

Amorphigenin generated a highly negative effect on osteoclastogenesis. We measured the effects of amorphigenin on BMMs with the XTT assay to exclude the possibility that the inhibition was due to cytotoxicity. amorphigenin demonstrated no cytotoxic effects at the same doses which effectively inhibited osteoclast differentiation (Fig. 2). Our results suggest that osteoclast formation inhibited by amorphigenin was not due to toxic effects on BMMs.

Amorphigenin inhibits a variety of signals transduced by RANKL

RANKL activates a variety of signal transducers that are involved in osteoclastogenesis, including p38, JNK, ERK, MAPK, Akt, and transcription factor NF-κB (Boyle *et al.,* 2003), which are recognized as the key factors of osteoclast differentiation. Osteoclast precursors were pretreated with amorphigenin and stimulated with RANKL at various time points. Different signaling pathways were observed. Phosphorylation of p38, Akt, and I-κB by RANKL were significantly inhibited by amorphigenin, but amorphigenin did not affect the phosphorylation of JNK and ERK (Fig. 3). This raises the possibility that the negative effect of amorphigenin is dependent on its inhibitory effect of certain signal transduction molecules such as p38 and NF-κB.
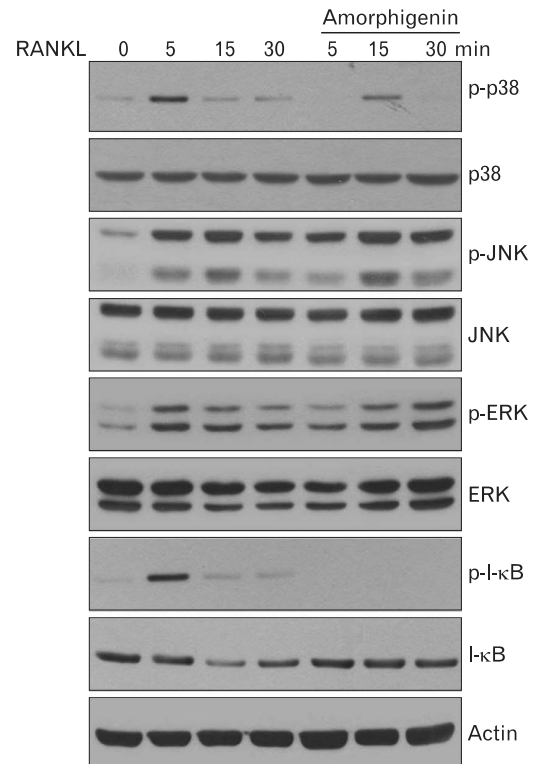

Fig. 3. Effects of amorphigenin in RANKL signaling. BMMs were pretreated with or without amorphigenin and stimulated with RANKL (100 ng/ml) for the indicated times. The cells were lysed in lysis buffer. Cell lysates were performed by Wesern blotting with the indicated antibodies.

Amorphigenin decreased the RANKL induced mRNA and protein levels of c-fos, NFATc1, TRAP, and OSCAR

We know from numerous previous reports that c-Fos and NFATc1 play a vital role in osteoclast differentiation. We examined the potential effects of amorphigenin on the expression of c-Fos and NFATc1 in response to RANKL. As shown in Fig. 4, amorphigenin inhibited the expression of c-Fos, NFATc1 and OSCAR mRNA expression in response to RANKL. Therefore, c-Fos and NFATc1 may be targets of amorphigenin in the inhibition of osteoclast differentiation induced by RANKL.

Amorphigenin suppresses the protein expression of c-fos and NFATc1 induced by RANKL

To elucidate the inhibitory effect of amorphigenin in mRNA expression, we next examined whether amorphigenin inhibits the protein levels of c-Fos and NFATc1 induced by RANKL, as shown by RT-PCR results. BMMs were treated with RANKL in the presence or absence of amorphigenin.
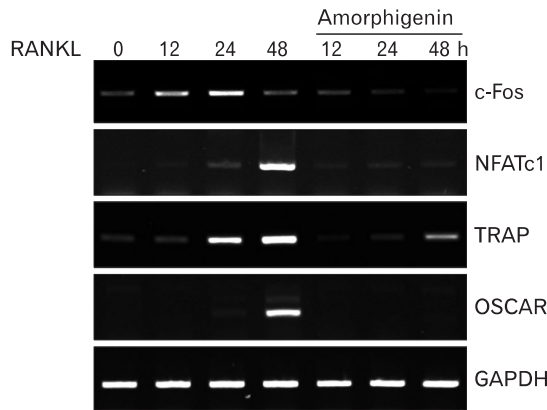
Fig. 4. Amorphigenin suppresses RANKL-induced gene expression. BMMs were pretreated with or without amorphigenin and then stimulated with RANKL (100 ng/ml) for the indicated times. Total RNA was isolated from the treated cells. The mRNA expression of c-fos, NFATc1, TRAP, OSCAR, and GAPDH was analyzed by RT-PCR.
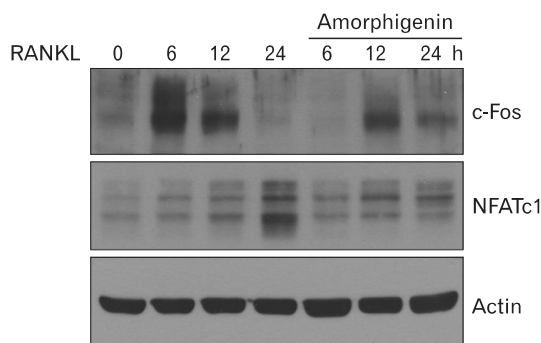

Fig. 5. Amorphigenin inhibits RANKL-mediated c-Fos and NFATc1 expression. BMMs were pretreated with or without amorphigenin and stimulated with RANKL (100 ng/ml) for the indicated times. The cells were lysed in lysis buffer. c-Fos, NFATc1, or actin proteins were detected by Western blotting with an anti-c-fos, and anti-NFATc1, or anti-actin-antibody, respectively.

Protein levels of c-Fos and NFATc1 were increased in BMMs treated with RANKL, as shown by RT-PCR analysis. However, the expression of both c-Fos and NFATc1 protein was significantly inhibited by amorphigenin (Fig. 5). These results suggest that the inhibitory effect of amorphigenin may be involved in the inhibition of c-Fos and NFATc1 induced by RANKL.

Effect of amorphigenin on LPS-Induced bone erosion in vivo

Osteoclasts are responsible for bone destruction in inflammatory bone disease, and inhibition of osteoclast differentiation by amorphigenin treatment may exert anti-
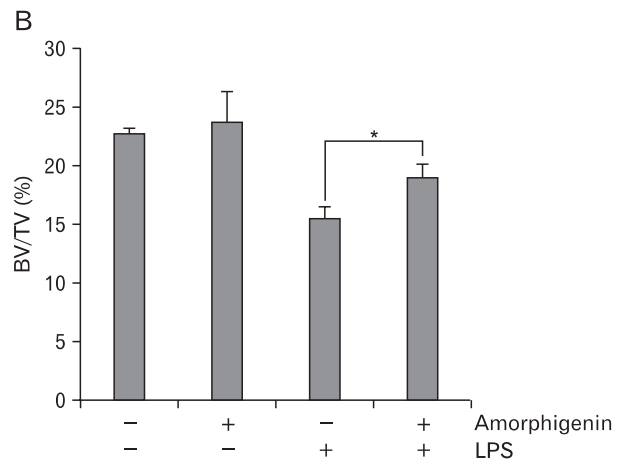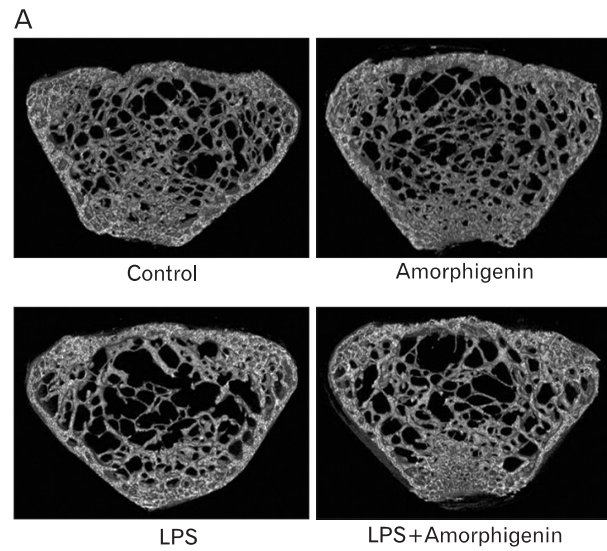

Fig. 6. Suppression of LPS-induced bone loss by amorphigenin. Mice were injected intraperitonealy with amorphigenin (10 μg/g of body weight) on days − 1, 1, 3, 5, and 7 days. LPS was injected intraperitoneally on days 0 and 4. The mice were killed 8 days after the first injection of LPS, and femurs were collected. (A) The femurs were imaged with a micro-CT machine. (B) Bone volume/tissue volume (BV/TV) was analyzed using the histomorphometric results. Asterisks indicate statistical difference between the indicated groups.

bone resorptive effect in vivo. Therefore to examine the effect of amorphigenin on bone erosion in vivo, an inflammatory bone disease model was created by intraperitoneal injection of LPS into mice. Oral administration of DW with or without amorphigenin was performed. On micro-CT scan, LPS injection induced severe trabecular bone loss in the femur. On the contrary, scans from mice in the oral amorphigenin-treated group showed significant reduction of bone loss (Fig. 6A). Furthermore, loss of bone volume (bone volume/tissue volume) induced by LPS was prevented in amorphigenin-administered mice (Fig. 6B).

Discussion

Bone is restructured at such a high speed that approximately 10% of the total bone content is replaced every year in human adults (Rodan & Martin, 2000). This balance is tightly regulated by numerous factors, and the most important factor is RANKL. Any tip towards one side of this balance will lead to osteoporosis or osteopetrosis (Walsh *et al.,* 2006). Most of the bone related metabolic disease has osteoclast as a main executor. Either a hyperactive or an inactive osteoclast is responsible for osteoporosis, inflammatory bone disease such as rheumatoid arthritis and periodontitis, paget disease and osteopetrosis (Bartold *et al.,* 2005; Everts *et al.,* 2009). Therefore, a compound to increase osteoblast activity and inhibit osteoclast activity would show potential as an ideal therapeutic for osteoporosis. Here we investigated the effect of amorphigenin in osteoclast differentiation that is derived from the leaves of *Amopha Fruticosa*. Amorphigenin considerably inhibited RANKL-induced osteoclast differentiation from BMMs in a dose-dependent manner without toxicity (Figs. 1 and 2).

The genes responsible for the differentiation of osteoclasts are expressed in different stages of development. In the early stage, PU.1 regulates differentiation of primitive hematopoietic cells (Hayashi *et al.,* 1998). M-CSF is responsible for inducing RANK in the surface of the hematopoietic stem cells, along with Bcl-2 for the regulation of survival and apoptosis (Zhao *et al.,* 2007). RANKL, RANK, TRAF6, NF-κB, NFATc1, c-Fos, and MITF are the major contributors for the differentiation of TRAP-positive mononuclear osteoclasts (Boyle *et al.,* 2003; Teitelbaum & Ross, 2003; Takayanagi 2005). And the key transcription factor is NFATc1, for this is the master regulator of osteoclast differentiation (Takayanagi *et al.,* 2002). NFATc1 transcribes DC-STAMP and Atp6v0d2 for cell fusion, cathepsin K, TRAP, OSCAR, MMP9 etc for function (Takayanagi 2007; Kim *et al.,* 2008). We also found that amorphigenin significantly inhibited expression of c-Fos and NFATc1 induced by RANKL, and the mRNA expression levels of markers of osteoclast such as TRAP and OSCAR was also inhibited by amorphigenin (Figs. 4 and 5). These results suggest that the differentiating mechanism of osteoclast differentiation influenced by amorphigenin is related to decreased expression of c-Fos and NFATc1.

LPS, a potent inducer of inflammation in vivo, induced bone erosion, although the mechanism of this effect remains unknown (Miyaura *et al.,* 2003). We previously have shown that LPS greatly induce bone erosion in vivo (Kwak *et al.,* 2010). Thus, we examined the effect of amorphigenin in LPS-mediated bone erosion. Importantly, LPS-injected mice showed a profound decrease in the density of trabeculae in the femur. However, amorphigenin significantly protected bone erosion induced by LPS (Fig. 6). Our results indicated that amorphigenin inhibits osteoclastic bone resorption as well as differentiation into osteoclasts in vivo.

The mainline drug that is currently used in the treatment of osteoporosis is bisphosphonates. The main function of bisphophonates is the binding of the drug to the hydroxyapatite of the bone, this can distort the absorption capacity of the osteoclasts (Russell *et al.,* 1999). Osteoclasts can also take this drug into their system, thus triggering an automated cell death system and inducing osteoclast apoptosis. Recent reports of bisphosphonates-related osteonecrosis of the jaw (Filleul *et al.,* 2010) and other adverse effects can limit the use of bisphosphonates. The demand for new drugs other than the traditional treatment modalities has constantly been up for grabs. Therefore, we assert that amorphigenin could be used not only for the treatment of post-menopausal osteoporosis and senile osteoporosis but also in numerous other areas such as in inflammatory arthritis and infectious conditions of bone such as osteomyelitis and chronic periodontitis.

Acknowledgements

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